

REMINERALIZING POTENTIAL AND ANTIBACTERIAL EFFECT OF DIFFERENT MOUTHWASHES ON DEMINERALIZED ENAMEL IN ACIDIC CHALLENGES: AN INVITRO STUDY

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ABSTRACT

Aim: Compare remineralizing potential and antibacterial effect of different mouthwashes applied to artificially demineralized enamel in acidic challenges.

Materials and methods: A total of 20 specimens were used and divided into four main groups according to the mouthwash used: Group 1: Listerine; Total Care Teeth Protect, Group 2: Listerine; Total Care Teeth & Gum Defense Group 3: DG WASH (Fluoride mouthwash) and Group 4: no mouth (control group). To evaluate remineralizing potential: first all specimens were exposed to 37.5% phosphoric acid for 90 seconds to promote the demineralization of enamel then followed by application of different selected mouthwashes to initiating re-mineralization process. All groups undergo acidic challenge by repeated cycles of de-mineralization and re-mineralization then tested for surface micro hardness at 3 different time intervals. The selected re-mineralizing solutions were assessed for their antibacterial effect against streptococcus mutans by using zone of inhibition test at 24, 48 hours.

Results: Micro hardness; there was a significant difference between tested groups with Listerine GP and saliva groups having significantly higher values than Listerine TP while for other intervals the difference was not significant. For DG wash, there was significant difference between different intervals. Bacterial test; there was a significant difference between tested groups with Listerine TP and Listerine GP having significantly higher values than other groups and with DG wash having significantly higher value than saliva group.

Conclusions: Combination of fluoride and essential oils has a synergetic effect of both actions regarding the re-mineralization and antibacterial potentially as compared to their effect alone.

KEYWORDS: Micro-hardness, Antibacterial Effect, Fluoride, Listerine, Essential oils

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INTRODUCTION

Over 21st century a lot of dental researches have advanced our conception of the etiology and pathogenesis of carious lesions. Also improving awareness of the dynamic process of demineralization/re-mineralization which has drove us to that supplying a steady low levels of intra-oral preventive methods as fluoride at the plaque, saliva or enamel interface, is proved to be one of the most effective method in dental caries prevention⁽¹⁾. One of the most approved efficient agent that has been used for long decades for caries prevention is fluoride.

Fluoride available in different forms of dental care products such as mouth washes, tooth pastes, gels, etc. Usually, patients with high caries index were advised to use a fluoride containing mouthwash and brush-on gel at home, as an adjunct to brushing with a fluoride tooth paste⁽²⁾. Besides the caries prevention role of fluoride, it also has a great impact in enhancing the re-mineralization of demineralized enamel that explain his effect in reducing the progress of initial carious enamel lesions⁽³⁾. In addition fluoride has a significant inhibitory effect on the cariogenic bacteria especially streptococcus mutans⁽⁴⁾.

Although fluoride still remains the cornerstone of modern non-invasive preventive method for dental caries lesions but emerging methods and agents, which can be used as alternatives to fluoride, have been introduced to the market and provide that they have anti-bacterial and or re-mineralizing action like; Chlorhexidine. Bioactive materials and some of natural essential oils⁽⁵⁾. Therapeutic mouthwash can improve oral hygiene by, decreasing the formation of dental plaque. Anti-plaque property of mouthwash is partly related to its antimicrobial capability, and the antiseptic ingredients as chlorhexidine and essential oils. Chlorhexidine is an evidenced based robust antibacterial effect through attaching and perforating cell membranes⁽⁶⁾. Unfortunately, CHX mouthwash reported some

ADRs, even at low concentrations contained taste alteration, discoloration of tongue and extrinsic tooth staining, numbness in mouth and tongue especially in long-term use⁽⁷⁾.

Antibacterial action of the essential oils mouthwash has been certified⁽⁸⁾. Listerine is a type of essential oils category and is considered to be one of the popular mouthwashes that is recommended by many dentists to be included in the daily dental care routine after brushing the teeth to maintain good oral health. Manufacture claimed that Listerine mouthwash could inhibits the formation of dental plaque subsequently will reduce the number of cariogenic bacterial in the oral cavity⁽⁹⁾. Listerine contains four plant-derived essential oils (eucalyptol, menthol, methyl salicylate, thymol), has also been shown to reduce plaque formation. Recently the Listerine manufacture introduce a new line of mouth washes containing the main essential oils of old version of Listerine conjugated with fluoride to enhance not its antibacterial action but also provide re-mineralizing effect. So the aim of the present study was to compare and asses the antibacterial effect and re-mineralizing potential of different mouthwashes applied to artificially demineralized enamel in acidic challenges.

Null hypothesis: Listerine mouth washes will have same re-mineralizing potential and antibacterial effect on de-mineralized enamel like fluoride mouthwash alone.

MATERIALS AND METHODS

Mouthwashes used in this study:

- **Listerine; Total Care Teeth Protect Milder Taste Mouthwash 250ml** (3 essential oils eucalyptol, menthol, methyl salicylate, thymol) and sodium fluoride.
- **Listerine; Total care Teeth & Gum Defense, Milder Taste, Soft Mint, 250ml** (3 essential oils eucalyptol, menthol, methyl salicylate, thymol) and sodium fluoride.

- **DG WASH:** Fluoride mouthwash.

Sample size calculation

The sample size was calculated with a probability (power) 0.9 according to previous study⁽¹⁰⁾ four experimental subjects and four control subjects were needed to be able to reject null hypothesis. For compensating any loss of the samples drop out, sample size was increased to 20% for each group (n = 5). Type I error probability was associated with test of null hypothesis is 0.5

Sample's preparation: The sample consisted of 20 specimens prepared from ten extracted sound human premolars were adopted for this present study. Teeth have been extracted either for orthodontic reasons or for periodontal diseases according to the ethical regulations for manipulation of extracted teeth by the research ethics committee of the faculty. Teeth were washed properly under running tap water and then remove the plaque or soft tissue remnants by polished of the teeth using fluoride-free polishing paste. Teeth were checked under a stereomicroscope to guarantee that they were not have any cracks, fractures or defects. Finally, they were stored in artificial saliva solution at room temperature.

Specimen's preparation: the teeth were sectioned in a mesio-distal direction into buccal and lingual halves using a low speed diamond cutting disc with water coolant. For facilitating the handling of the specimens throughout the different steps of the experiment each half was embedded in chemically cured acrylic resin blocks while keeping enamel surface uncovered. The exposed surfaces were polished by using So-Lex discs with different abrasives level; medium, fine and extra fine (3M) to make certain that the aprismatic enamel is took off⁽¹¹⁾.

Grouping of the specimen and study design: The specimens were divided into four main testing groups (fives teeth each) according to the mouthwash used in each one. **Group 1:** Listerine; Total Care Teeth Protect, **Group 2:** Listerine;

Total Care Teeth & Gum Defense, **Group 3:** DG WASH (Fluoride mouthwash) and **Group 4:** no mouthwash. Each group was stored in artificial saliva ready for the *baseline micro-hardness values* before going through the enamel demineralization. Artificial saliva was prepared at the Laboratory of Biochemistry, Faculty of Pharmacy, Cairo University, Egypt. Artificial saliva was prepared according to the formulation of Ten Cate and Duijsters⁽¹²⁾ which contained 1.5mM CaCl₂, 0.9mM NaH₂ PO₄, 0.15M KCl (potassium chloride) at pH 7.0

De-mineralization of Enamel: immersing all the specimens in 37.5% phosphoric acid for 90 seconds to promote the demineralization of enamel and then rinsed well with water and gently air dried⁽¹³⁾. Now the specimens ready for the *second readings* of micro-hardness after demineralization procedure.

Re-mineralization protocols: in all three first tested groups mouthwashes were applied as following prescribed protocol. Group 1: Listerine; Total Care Teeth Protect (15mL), Group 2: Listerine; Total Care Teeth & Gum Defense (15mL) and Group 3: DG WASH (Fluoride mouthwash) (15mL) were used to totally immersed the specimens after removal from artificial saliva and left in the mouthwash for 30 seconds; according to the manufacture instructions; and then specimens re-immersed in renewed artificial saliva. This procedure was repeated twice a day for 14 days. While for Group 4: no mouthwash was used and the specimens kept in the artificial saliva which changed twice a day as the rest of all the tested groups.

Acidic challenge protocol: all the tested groups are exposed to de-mineralization and Re-mineralization cycles. For re-mineralization cycle; using the different tested mouthwashes for the first three groups and used artificial saliva for the 4th group. While for the de-mineralization cycle; a demineralizing solution was prepared of pH 4 which consists of 133 mmol/L NaCl and 50mmol/L

lactic acid. A de-mineralization cycle and a re-mineralization cycle were repeated daily for 14 days for all the tested groups by immersing the specimens in this de-mineralizing solution (15 mL) for 1 hour. This protocol is almost equivalent to the cumulative. Acidic challenge times of 24-hours. Period inside the oral cavity⁽¹¹⁾. After passing of 14 days all specimens were ready for the **third and final readings** of micro-hardness.

Assessment of Surface Micro-hardness: micro-hardness was measured at baseline of sound enamel, after de-mineralization and after 14 days. Surface Micro-hardness of the specimens was determined using Digital Display Vickers Micro-hardness Tester (Model HVS-50, Laizhou Huayin Testing Instrument Co., Ltd. China) with a Vickers diamond indenter and a 20X objective lens. A load of 100g was applied to the surface of the specimens for 15 seconds. Three indentations, which were equally placed over a circle and not closer than 0.5 mm to the adjacent indentations, were made on the surface of each specimen (14). The diagonals length of the indentations were measured by built in scaled microscope and Vickers values were converted into micro-hardness values. Micro-hardness calculation; Micro-hardness was obtained using the following equation:

$$HV=1.854 P/d^2$$

where, HV is Vickers hardness in Kgf/mm², P is the load in Kgf and d is the length of the diagonals in mm.

Antibacterial assessment: Agar Plate Diffusion Test (Zone of inhibition test) was used to evaluate the antibacterial effect of the three different tested mouthwashes against the streptococcus mutans (S. mutans). S. mutans (UA159) was obtained from the culture stock of the Department of Microbiology and Immunology of Cairo University. The indicator strain was first grown on Mitis salivarius agar plates at 37 °C for 48 h in a 10% CO₂ incubator (BBL Gas Pak, Becton Dickinson USA). Subsequently, single colonies were inoculated into 5 mL of Brain Heart Infusion (BHI) broth and incubated at 37 °C for 24

h to form a suspension (inoculum). In each sterilized Petri dish (20x100 mm), a base layer containing 15 mL of BHI agar mixed with 300 mL of each inoculum was prepared. After solidification of the culture medium, we have three agar plates for each one 50 microns of each mouthwash were placed in the agar plate in certain area that was marked for each material figure (1). The plates were incubated at 37 °C for 24 hours, (all procedures carried out at anaerobic jar). Zones of bacterial growth inhibition were recorded in millimeters (mm) using a digital caliper. Measurements were taken at the greatest distance between two points at the outer limit of the inhibition halo formed around the specimen^(15, 16). This measurement was taken after 24 hours, 48 hours



Fig. (1) Showing inhibition zones around different tested mouthwashes'. V: Listerine; Total Care Teeth Protect, G: Listerine; Teeth & Gum Defense, D: DG Wash, S: Artificial saliva

A mean and standard deviation for the Measurements of both surface Micro-hardness and Microbiological test were determined per group.

Statistical analysis

Numerical data was represented as mean and standard deviation (SD) values. Shapiro-Wilk's test was used to test for normality. Micro-hardness data were normally distributed and were analyzed using one-way ANOVA followed by Tukey's post hoc test for intergroup comparisons and repeated measures ANOVA followed by Bonferroni post hoc test for intragroup comparisons. Inhibition zones data were

non-parametric and were analyzed using Kruskal-Wallis test followed by Dunn’s post hoc test for inter group comparisons and signed rank test for intragroup comparisons. P-values were corrected for multiple comparisons using Bonferroni correction. The significance level was set at $p < 0.05$ within all tests. Statistical analysis was performed with R statistical analysis software version 4.2.3 for Windows.

RESULTS

Results of inter and intragroup comparisons for micro-hardness are presented in table (1). Results showed that for measurements taken at the demineralization stage, there was a significant difference between tested groups with Listerine GP and saliva groups having significantly higher values than Listerine TP ($p = 0.043$). For other intervals, the difference was not statistically significant ($p > 0.05$). For DG wash, there was a significant difference

between values measured at different intervals with baseline value being significantly higher than that of demineralization ($p = 0.037$). For other groups, there was no significant difference between values measured at different intervals ($p > 0.05$). Mean and standard deviation values for micro-hardness are presented in figures (2) and (3).

Results of inter and intragroup comparisons for bacterial inhibition zones are presented in table (2). Results showed that for both intervals, there was a significant difference between tested groups with Listerine TP and Listerine GP having significantly higher values than other groups and with DG wash having significantly higher value than saliva group ($p > 0.05$). For all groups, there was no significant difference between values measured at both intervals ($p > 0.05$). Mean and standard deviation values for bacterial inhibition zones are presented in figures (4) and (5).

TABLE (1) Inter and intragroup comparisons of micro-hardness

Time	Group	Micro-hardness (Mean±SD)				f-value	p-value
		Listerine TP	Listerine GP	DG wash	Saliva		
Baseline		294.61±13.62 ^{Aa}	310.52±3.03 ^{Aa}	304.20±6.40 ^{Aa}	289.16±9.87 ^{Aa}	3.30	0.079
Demineralization		288.17±8.91 ^{Ba}	306.86±4.07 ^{Aa}	296.61±7.23 ^{ABb}	301.99±5.32 ^{ABa}	4.36	0.043*
Remineralization		298.82±4.46 ^{Aa}	308.07±2.95 ^{Aa}	299.33±6.82 ^{Aab}	300.94±2.05 ^{Aa}	2.78	0.110
f-value		1.20	5.78	8.38	3.52		
p-value		0.391	0.066	0.037*	0.131		

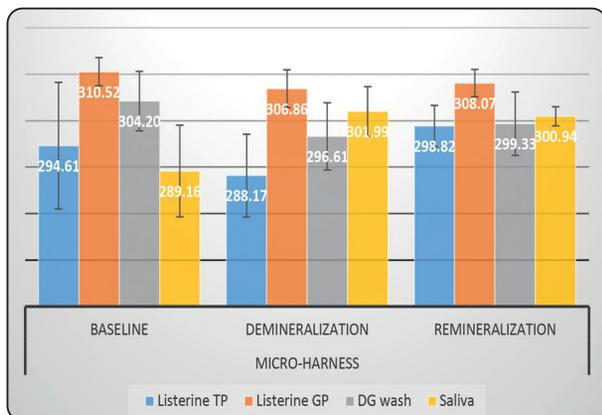


Fig. (2) Bar chart showing mean and standard deviation values (error bars) for micro-hardness

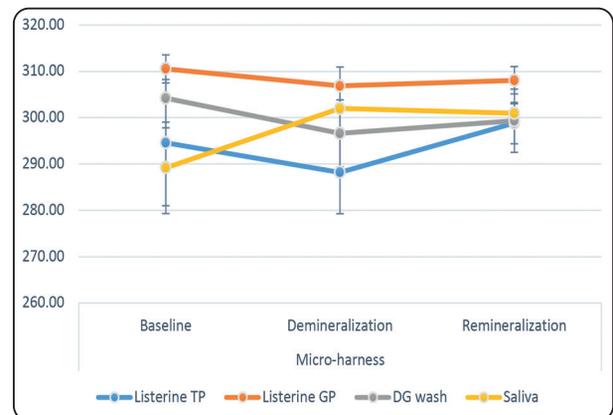


Fig. (3) Line chart showing mean and standard deviation values (error bars) for micro-hardness

TABLE (2) Inter and intragroup comparisons of bacterial inhibition zones (mm)

Time	Group	Bacterial inhibition zones (mm) (Mean±SD)				h-value	p-value
		Listerine TP	Listerine GP	DG wash	Saliva		
24 hours		30.00±5.00 ^A	37.33±6.43 ^A	23.00±6.93 ^B	0.00±0.00 ^C	9.13	<0.001*
48 hours		33.33±3.51 ^A	39.33±6.03 ^A	17.67±4.04 ^B	0.00±0.00 ^C		
	<i>u-value</i>	6.00	3.00	6.00	NA		
	<i>p-value</i>	0.250	0.346	0.174	NA		

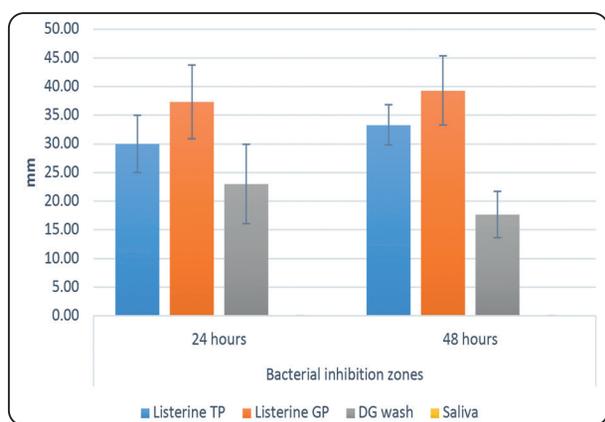


Fig. (4) Bar chart showing mean and standard deviation values (error bars) for bacterial inhibition zones (mm)

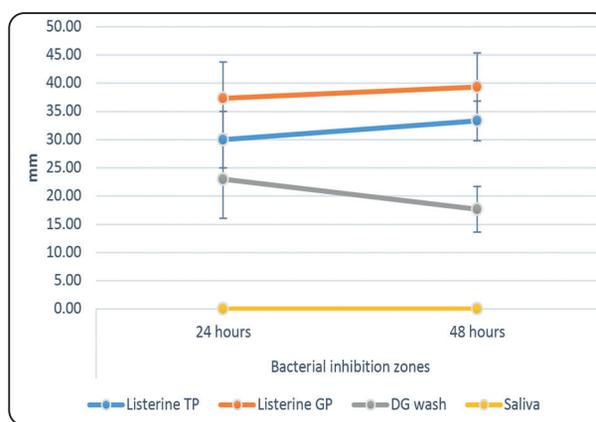


Fig. (5) Line chart showing mean and standard deviation values (error bars) for bacterial inhibition zones (mm)

DISCUSSION

Oral environment has many continuous struggling facing the natural tooth structures like biological, thermal and mechanical ones. The capability of enamel to withstand against these challenges is the balance existing between these different continuous oral environmental changes. Acidic challenge is one of this struggle that has a deleterious effect on tooth structure⁽¹⁷⁾. As we all know that, prevention is better than cure, so many researches were conducted on various re-mineralizing techniques and agents that could help in preventing tooth de-mineralization or restoring the lost minerals^(13,18).

Dental caries is a multifactorial disease that at most produced due to loss of minerals of enamel and dentin in conjunction with acid production of cariogenic bacteria especially, streptococcus mutans, which lead to destruction of the organic

matrix^(15,19). Topical application of dental agents that have antibacterial and/or re-mineralizing effect will obviously reducing the incidence of dental caries by enhancing the re-mineralization with provide suitable oral environment PH through decreasing the number and activity of the cariogenic bacteria^(5,8,13). This would explain the value of this present study, which aimed to compare the antibacterial Effect and Re-mineralizing Potential of different mouthwashes applied to artificially demineralized enamel in acidic challenges.

Fluoride is a "Hero" of preventive and minimal invasive dentistry as its potent antibacterial and re-mineralizing agent with strong evidence-based data about its impact on minimizing the incidence of dental carious when used in any topical form⁽²⁰⁻²³⁾. But Lobo PL et al., 2008 mentioned that the clinical use of fluoride and administration of it as a carious

preventive agent requires using large concentrations with continuous frequency of application to go beyond the concentration prerequisite to decrease enamel demineralization as a result of this patients may go through some health problems as fluorosis⁽²⁴⁾. Therefore; advanced researches were conducted to find other natural alternatives agents that having at least the same results of these chemical agents to reducing its application with their side effects.

One of these researches is to find planet-based extracts as essential oils that have a curing effect on many dental lesions^(5,9) these trials and researches depend on what is called "PHYTOTHERAPY". In this present study we chose 2 types of Listerine mouthwashes which consists mainly from at least 3 essential oils to compare its effect on re-mineralizing and antimicrobial effect alone or when conjugated with fluoride to explore if the combination of these essential oils and fluoride will produce synergetic effect or antagonist effect to fluoride action. So we used Listerine; Total Care Teeth Protect, Listerine; Mouthwash, Teeth & Gum Defense and DG WASH (only Fluoride mouthwash).

To assess the re-mineralizing effect and the antibacterial effect of both intervals (Listerine and Fluoride) we were have been used Vickers micro-hardness test and Zone inhibition test. Loss or gaining minerals of enamel could be assessed by various techniques; one of these technique is surface micro-hardness assessment as their values change greatly upon mineral loss or gain^(14,15). All specimens were assessed at baseline, after enamel de-mineralization and after 14 days of repeated cycles. Assessments of each specimen at various platforms during the experiment provide us with reliable correct foretelling about how many minerals lost or gained by the tooth structure throughout the study⁽²⁵⁾. Inter-groups comparison revealed that after demineralization phase there was reduction in micro-hardness due to loss of minerals from enamel with significant difference among the groups which my attributed to variation of original minerals content of each specimen. Obtained values reported

statistically significant increasing in surface micro-hardness recording high values for Listerine total care, saliva and Listerine teeth and gum respectively as compared with DG mouthwash group.

This result was in agreement with antibacterial results for the same groups except that for artificial saliva that showed re-mineralizing potentially more significant than antibacterial effect. Which could be attributed synergism influence that obtained by integration of fluoride with essential oils of Listerine mouthwashes that furthermore documented by⁽²⁶⁾ reported fluoride mouthwashes with essential oils have remarkable increasing in minerals uptake when compared to an essential oil non-fluoride mouthwash or fluoride non-essential oils mouthwashes. Finally according to the results the null hypothesis of this study was totally accepted.

The antibacterial effect was examined with respect to *Streptococcus mutans*, which is documented to be the most relevant caries-related micro-organism⁽²⁷⁾. Zone inhibition test which also called 'Agar Plate's diffusion method' was adopted for this study as it was recorded that this method allow prolonged antibacterial activity of any materials by the direct contact between bacteria and tested material^(27,28). Antimicrobial assessment test revealed the at 24 and 48 hours recorded statistically significant reduction the streptococcus mutans especially for the s Listerine mouthwashes followed by DG wash and finally antiradical saliva group which may be explained by the synergetic effect that obtained by combination of essential oils^(5,9,29) with fluoride^(1,2,3,15) as both materials was documented that it has an potent antibacterial effect.

This result was in coordination with the result of Kato et al., 1999 who reported a decrease of not only the number of microorganisms and decrease in plaque induced by mouth washing in response to Listerine. In addition; thymol oil one of the essential oils contained in Listerine that possesses various beneficial effects due to its antiseptic, antimicrobial, and anti-oxidative properties⁽³⁰⁾. Also Eucalyptus

oil, there are many recorded data confirmed the antimicrobial activity of it toward oral bacteria⁽³¹⁾. Finally according to the results the null hypothesis of this study was totally accepted.

CONCLUSION

Under the limitations of this study, the following conclusions can be derived:

1. Listerine mouthwashes with different formula have impact role in enhancing re-mineralization of de-mineralized enamel and decreasing the number of streptococcus mutans.
2. Combination of fluoride and essential oils has a synergetic effect of both actions regarding the re-mineralization and antibacterial potentially as compared to their effect alone.

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